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㉖ Synthetic phospholipid compounds, their preparation and use.

㉗ Synthetic phospholipid compounds which are phosphatidylethanolamine polycarboxyl polyalkylene glycols are prepared by the reaction of a phosphatidylethanolamine, a polycarboxylic acid compound, such as succinic or glutaryl anhydride, and a polyalkylene glycol, such as polyethylene glycol.

- 1 -

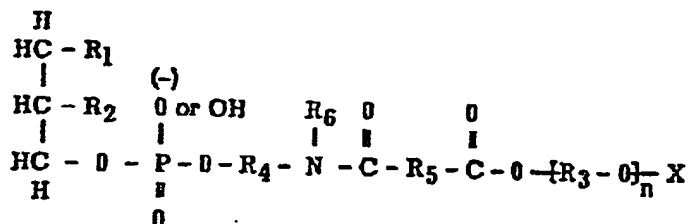
SYNTHETIC PHOSPHOLIPID COMPOUNDS, THEIR PREPARATION AND USE

- Phospholipids such as lecithin, are amphiphatic compounds in that they contain both hydrophobic and hydrophilic groups or regions within the same molecule. The balance between these hydrophobic and hydrophilic regions determines the physical properties of the compounds in an aqueous environment. The uses of natural phospholipids as additives are numerous in the food industry (for example, as emulsifiers), in cosmetics, for industrial uses and for the pharmaceutical industry, especially in the preparation of drug-delivery systems. U.S. Patents 4,086,257; 4,097,502; 4,145,410; and 4,159,988 disclose various modifications of the polar-head-group region of natural phospholipids which lead to unique and unexpected physical properties.
- Further, various derivatives of lecithin are known, such as, for example, oxyalkylated lecithin compounds (see U.S. Patents 3,085,100 and 2,310,679) and phosphatidylalkanolamine derivatives (see, for example, U.S. Patents 2,801,255; 3,542,820; 3,577,466; and 4,254,115). It is desirable to provide novel, synthetic phospholipids, particularly having enhanced solubility and surfactant properties in an aqueous environment, especially for the formulation of water-insoluble materials, such as drugs or cosmetic ingredients, within an aqueous environment.
- European Patent Application 0072111 (published 16 February 1983) describes novel, synthetic phospholipid compounds, such as phosphatidylalkanolamine carboxyl polyalkylene glycol compounds, including phosphatidylethanolamine carboxyl polyethylene glycol compounds. These compounds are prepared by the covalent reaction of a carboxylic analog of the polyalkylene glycol with the phosphatidylalkanolamine, to provide biodegradable, phospholipid compounds which contain an amide linkage.

- 2 -

This invention relates to synthetic phospholipid compounds, to their preparation and to the use of such compounds, particularly in solubilizing, in an aqueous environment, water-insoluble compounds.

- 5 The invention describes a new, improved series of phospholipid compounds in which the polar-head-group region is modified by the covalent attachment through two or more carboxylic groups of polyalkylene glycols. The novel, synthetic phospholipid compounds of the invention are
- 10 phosphatidylalkanamine polycarboxyl polyalkylene glycol compounds, such as phosphatidylethanolamine di- or tri-carboxyl polyethylene and polypropylene glycol compounds. The phospholipids of the invention are analogs of the phospholipid compounds of European Patent Application
- 15 0072111. This invention also provides a different and improved method of coupling a polyalkylene glycol to a phospholipid. The phospholipid compounds of the invention have enhanced surfactant properties, are soluble in acetone and are biodegradable.
- 20 The synthetic phospholipid compounds of the invention are represented by the following structural formula:



- 3 -

wherein

a)  $R_1$  and  $R_2$  each represent hydrogen or a saturated or unsaturated, straight or branch-chain acyl group, such as an organic acyl radical having 2 to 24 carbon atoms; for example,  $C_2$  to  $C_{20}$  fatty-acid radicals, such as oleic, stearic, linoleic, linolenic, palmitic, myristic or arachidonic acid radicals, or may be derived from natural products, such as plants like soybean or egg;

b)  $R_3$  represents an alkylene radical, such as a  $C_2$  to  $C_4$  group like ethylene, propylene or butylene; that is, tetramethylene;

c)  $R_4$  represents a polymethylene radical, typically a  $C_2$  to  $C_{10}$  polymethylene divalent radical, and particularly an ethylene radical; that is a dimethylene radical, as in natural lecithin, or a propylene radical; that is a trimethylene radical;

d)  $R_5$  represents an organic linking radical; for example, a hydrocarbon radical, of from 1 to 24 carbon atoms, but typically of 2, 3 or 4 carbon atoms, which radical may be saturated or unsaturated, may be substituted, for example, with hydroxyl, amino or carboxyl groups, or be an unsubstituted radical, such as a polymethylene ( $-CH_2-$ ) radical like an ethylene, propylene or butylene radical;

e)  $R_6$  represents hydrogen or an alkyl, such as methyl, radical;

f)  $X$  represents hydrogen or an alkyl radical, typically a lower alkyl radical like a  $C_1$  to  $C_4$  alkyl group, such as a methyl radical; and

g)  $n$  represents a number of the alkylene oxide groups and may vary from 0 to about 200 or more; for example, from 2 to 100, such as 2 to 20, to provide phospholipids having, for example, a molecular weight of from 150 to 3000 or more; for example, 200 to 2000.

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The polycarboxylic acids or derivatives used in the preparation of the phospholipid compounds are preferably aliphatic acids and more particularly aliphatic acid anhydride compounds for ease of reaction; for example,  $C_3$  to  $C_6$  dicarboxylic acids, particularly which form 4-to-7-member-ring anhydrides, such as succinic acid, glutaric acid, adipic acid and phthalic acid anhydride. Suitable carboxylic acids include, but are not limited to, aliphatic, cycloaliphatic, di- and tri-carboxylic acids, such as succinic acid, glutaric acid, glutamic acid, citric acid, tartaric acid, oxalic acid, adipic acid, malic acid and maleic acid, as well as long-chain dicarboxylic acid, although waxy, solid compounds may result from the use of long-chain acids. Preferably the acid compounds employed

- 5 -

are the C<sub>3</sub> to C<sub>7</sub> dicarboxylic acid anhydrides. These novel phospholipid compounds have a distinctly different chemical composition than the compounds described, for example, in U.S. Patents 2,310,679 and 3,085,100, which are products from the coupling of ethylene oxide or similar compounds to crude soy lecithin.

The use of the term "lecithin" describes a number of compounds including lecithin (that is, phosphatidylcholine), a compound that cannot react with ethylene oxide. On the other hand, soy lecithin does contain phosphatidylethanolamine, phosphatidylinositol and a variety of glycolipids. All of these compounds in crude lecithin can react with ethylene oxide or similar compounds containing a reactive cyclooxide group to form various adducts. For example, in phosphatidylinositol and with glycolipids, the reactive groups in these molecules are hydroxyl groups which will form an ether linkage, when reacted with ethylene oxide. Phosphatidylethanolamine, which contains a primary amino group, will react with ethylene oxide to form an alkylamine linkage (see N. Schonfeldt, "Surface Active Ethylene Oxide Adducts", Pergamon Press, 1969). In both cases, these adducts are not biologically degradable, and, therefore, such compounds are undesirable for use in the cosmetic and pharmaceutical industries.

The phospholipids of the invention comprise synthetic phospholipids in which the linkage between the synthetic ethylene oxide or propylene oxide polymer and the naturally occurring phospholipid is a biologically degradable linkage; for example, an amide linkage, which makes these novel phospholipid compounds useful for cosmetics and pharmaceutical uses.

- 6 -

The preparation of the phospholipid compounds is best accomplished by the addition of a cyclic polyacid, particularly anhydride, such as succinic or glutaric acid anhydride, to the polyalkylene oxide polymer. The coupling of the appropriate carboxylic analog of the polyalkylene oxide polymer to the phosphatidylalkanolamine molecule, such as phosphatidylethanolamine, gives the desired compounds. Alternatively, the cyclic acid anhydride can be coupled to the phosphatidylethanolamine, and then the polyalkylene oxide polymer is coupled to the modified phospholipid. For example, in one method the low-cost acidic polyalkylene glycol compound can be mixed with crude soy lecithin for a coupling reaction with the phosphatidylethanolamine, and the novel phospholipid compounds extracted with acetone. The acidic polymer may be converted to an acid halide compound, to increase the speed of the reaction. The acidic polyalkylene glycol polymer compound can be purified further via distillation, ion-exchange chromatography or absorption chromatography. The acyl analog of the polyethylene oxide polymer is activated by a convenient activating agent, such as oxalyl chloride or 1, 1 carbonyl diimidazole. The activated carboxylic derivative of the parent polyalkylene oxide polymer is then coupled to the phosphatidylethanolamine via an amide linkage, to form the phospholipid analog compounds of this invention.

The phosphatidylethanolamine either can be isolated from natural sources, synthesized according to established chemical procedures, or enzymatically synthesized using the corresponding phosphatidylcholine compound in the presence of ethanolamine and phospholipase D. The reaction of the phosphatidylethanolamine and the carboxylic derivative of the polyalkylene oxide polymer is carried out in an inert solvent. The progress of the reaction can be monitored by thin-layer chromatography. Purification of the final product, if necessary, may be carried out using column



- 7 -

chromatography.

In the phospholipid compounds of this invention, the polar-head group of the phosphatidyl ethanolamine has been modified to alter its physical properties, by the inclusion of a polyalkylene oxide polymer. In all cases where natural phospholipids can be used, such as in drug-delivery systems, in cosmetics, in food, in industrial uses, in treating atherosclerosis, for intravenous nutrition and other uses, these new synthetic phospholipid compounds can be used alone or in combination with other natural phospholipids, especially phosphatidylcholine. Biologically these synthetic phospholipids will be immunologically inert. For example, polyethylene oxide polymers attached to proteins are nonimmunogenic and well tolerated by the body (see Abuchowski et al, J. Biol. Chem. 252, pp 3578-3581 (1977)). The covalent linkage between a typical polyalkylene example, such as polyethylene oxide polymer and the phosphatidylethanolamine, is biologically degradable, and phosphatidylethanolamine, itself, is a natural compound.

As a result, these novel compounds have utility in encapsulating drugs, especially water-insoluble drugs, as ~~drug-delivery systems that either can be administered orally or via injection~~, such as in the encapsulation process disclosed in corresponding U.S. Serial No. 90,994, filed November 13, 1979, as well as in the method of U.S. Patent 4,016,100.

The presence of the hydrophilic alkylene oxide polymer, particularly the polyethylene oxide polymer moiety in the phospholipids, also gives rise to novel and unexpected physical properties in an aqueous environment. As an example, unsaturated phosphatidylethanolamines, especially those isolated from soybeans, do not form any stable type of structure in water. Phosphatidylcholine, if hydrated with an aqueous solution forms large (>200 nm or 2000 Å) structure.

- 8 -

termed multilamellar liposomes. On the other hand, gangliosides have a similar hydrophobic region, compared to phosphatidylethanolamine and phosphatidylcholine, but the polar region of the ganglioside molecule is composed of hydrophilic oligosaccharides. The presence of these oligosaccharides allows the ganglioside to organize into a stable micelle upon hydration with water. By covalently attaching a hydrophilic polyalkylene polymer, such as polyethylene or polypropylene oxide polymers, to phosphatidylethanolamine, a phospholipid analog to ganglioside is essentially synthesized. It also should be noted that, while no molecular species of phosphatidylethanolamine will form a stable structure in an aqueous environment, the phospholipid analog compounds described herein do form stable structures upon hydration. As a consequence of this physical behavior, a variety of water-insoluble compounds can be formulated in a stable form in an aqueous environment at physiological pH. Furthermore, the spontaneous structure that these phospholipids form, when hydrated with water from the dry state, is small (less than 20 nm or 200 Å; for example, typically average 7.5 to 10 nm or 75 to 100 Å), which results in an optically clear solution.

The actual organization of these structures, however, will depend, at least in part, on the selected acyl chain composition of the phosphatidylethanolamine and the alkylene oxide polymer. In particular, with phosphatidylethanolamine isolated from soybeans and various polyethylene oxide polymers, micellar structures of less than 20 nm (200 Å) diameter are spontaneously and easily formed, upon addition of water to the dried phospholipid analog. These structures are distinct and unique, as compared to liposomes or other lipid vehicles that are composed of phosphatidylcholine.

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- 9 -

The invention is illustrated by the following examples:

Example 1. Soy phosphatidyl thanolamine succinyl polyethylene glycol monomethyl ether (molecular weight 252)

- Succinyl polyethylene glycol monomethylether compounds
- 5 were prepared in accordance with and under the following general method and conditions. 1 mole of the polyethylene glycol polymer was heated with 5 moles of finely divided succinic anhydride in a slurry of  $\text{CCl}_4$  under a nitrogen atmosphere. The solution was magnetically stirred and heated
- 10 overnight at  $75^\circ\text{C}$  under a nitrogen atmosphere. Temperature control is important, as the reaction mixture darkens above  $75^\circ\text{C}$ . Upon cooling at room temperature, the excess succinic anhydride was removed by filtration. The reaction mixture was then dissolved in a 1:1 methylene chloride/methylene
- 15 mixture, and sufficient concentrated  $\text{NH}_4\text{OH}$  was added, so that an aliquot of the reaction mixture was approximately pH 9, when diluted with water. The succinyl polymer reaction mixture was dissolved in a 95:5:0.8 mixture of methylene chloride/methanol-concentrated  $\text{NH}_4\text{OH}$  and applied to a silicic
- 20 acid column equilibrated in the same solvent. The succinyl polymer was eluted with a step gradient of increasing methanol content. The purity of the fraction was monitored by thin-layer chromatography, using a solvent system of 90:10:1 of methylene chloride/methanol-concentrated  $\text{NH}_4\text{OH}$ .
- 25 Pure fractions of the succinyl polymer were evaporated to near dryness. Sufficient  $\text{HCl}$  was added to the mixture, so that an aliquot, when diluted with water, gave a pH of between 2 and 2.5. The compound was then evaporated to dryness. Methylene chloride was added to the dried material,
- 30 and any particulate material was filtered off. The filtrate was evaporated to dryness and dried overnight under high vacuum.

- 10 -

The above conditions were used to make the succinyl polyethylene glycol monomethylether, using, as the starting material, polyethylene glycol monomethylether with a molecular weight of 252. To 2200  $\mu$ moles of the succinyl polymer compound were added 2000  $\mu$ moles of 1,1 carbonyl diimidazole in a benzene solution. The solution was heated at 60°C for 10 to 15 minutes, until the bubbling had ceased. This solution was transferred to another flask containing 1000  $\mu$ moles of dried soy phosphatidylethanolamine (PE), which was prepared by the enzymatic conversion of soy phosphatidylcholine using ethanolamine and phospholipase D from Savoy cabbage. Sufficient benzene was added to ensure that all of the soy PE had dissolved. Then most of the benzene was removed, leaving a thick slurry. This slurry was stirred with a magnetic stirrer for approximately 6 hours at 70°C under a nitrogen atmosphere. The extent of the conversion of PE to the desired product was monitored by thin-layer chromatography. The reaction mixture was taken to dryness and then partitioned into a two-phase Folch System, with .2M HCl in the upper phase. The lower phase was extracted four more times with this acidic Folch upper phase, and twice with a .3M  $\text{NH}_4$  acetate upper phase, to remove the imidazole and excess noncoupled succinyl polymer compound. The lower phase was evaporated to dryness. The sample was dissolved in 95:5:0.8 methylene chloride/methanol-concentrated  $\text{NH}_4\text{OH}$  and applied to a silicic acid column equilibrated in the same solvent. The soy PE-succinyl polyethylene glycol monomethylether (molecular weight 252) was eluted with a step gradient of increasing methanol content, while maintaining approximately 0.8 to 1.0 concentrated  $\text{NH}_4\text{OH}$  in the eluting solvent. The elution profile was monitored by thin-layer chromatography, using 80:2:1 methylene chloride/methanol-concentrated  $\text{NH}_4\text{OH}$  as the solvent. Pure fractions of the product were pooled and

- 11 -

reduced to dryness. The pH was tested and adjusted, if needed, to between 5 and 7. The compound was then dissolved in methylene chloride. The yield of the product was 35% based on the starting soy PE content.

- 5 Example 2. Soy phosphatidylethanolamine succinyl polyethylene glycol monomethylether (molecular weight 120)

A succinyl polyethylene glycol monomethylether (molecular weight 120) polymer compound was prepared as described in Example 1. 7500  $\mu$ moles of the succinyl polyethylene glycol were dissolved in benzene and 4000  $\mu$ moles of 1,1 carbonyl diimidazole were added. The solution was heated at 60°C, until all bubbling had ceased. The solution was added to 1000  $\mu$ moles of soy PE that previously had been dried under high vacuum. The reaction conditions were the same as in Example 1. The yield of the pure product, after column chromatography, was 42%.

- 10 Example 3. Soy phosphatidylethanolamine succinyl polyethylene glycol monomethylether (average molecular weight 1900)

20 A succinyl polyethylene glycol monomethylether (average molecular weight 1900) was prepared as described in Example 1. 1750  $\mu$ moles of this compound were dissolved in benzene and 1000  $\mu$ moles of 1,1 carbonyl diimidazole were added. The solution was heated to 60°C, until the bubbling had ceased. The solution was added to 483  $\mu$ moles of dried soy PE. The reaction conditions were as described in Example 1. The yield of column-purified material was 44%.

- 25 Example 4. Soy phosphatidylethanolamine succinyl polyethylene glycol monomethylether (average molecular weight 350)

30 A succinyl polyethylene glycol monomethylether (average molecular weight 350) was prepared as described in Example 1. 7000  $\mu$ moles of succinyl polyethylene glycol monomethylether compound were dissolved in benzene and 4000  $\mu$ moles of 1,1 carbonyl diimidazole were added. The solution was heated at 60°C, until the bubbling had ceased. This solution was added

- 12 -

to 1050  $\mu$ moles of dried soy PE. The reaction conditions to form the product were the same as described in Example 1. The yield of the pure product, after column chromatography, was 70%.

- 5 Example 5. Soy phosphatidylethanolamine succinyl polyethylene glycol monomethylether (average molecular weight 750)

A succinyl polyethylene glycol monomethylether (average molecular weight 750) was prepared as described in Example  
10 1. 7500  $\mu$ moles of the compound were dissolved in benzene and 5000  $\mu$ moles of 1,1 carbonyl diimidazole were added. The solution was heated at 60°C, until bubbling had ceased. This solution was then added to 1200  $\mu$ moles of dried soy PE. The reaction conditions were then as described in Example 1. The  
15 yield of the column-purified product was 52%.

Example 6. Soy phosphatidylethanolamine succinyl polyethylene glycol monomethylether (average molecular weight 550)

A succinyl polyethylene glycol monomethylether (average molecular weight 550) was prepared as described in Example 1.  
20 3070  $\mu$ moles of the compound were dissolved in benzene and 2000  $\mu$ moles of 1,1 carbonyl diimidazole were added. The solution was heated at 60°C, until the bubbling ceased. The solution was added to 1127  $\mu$ moles of dried soy PE. The reaction conditions were the same as described in Example 1.  
25 The yield of the column-purified material was 58%.

Example 7. Soy phosphatidylethanolamine succinyl polyethylene glycol monomethylether (molecular weight 252)

Another method of producing the compounds of the invention is the coupling of succinic anhydride to soy PE and  
30 then reacting the modified PE with the appropriate polyethylene glycol polymer. Succinic anhydride (1000  $\mu$ moles) was reacted with 1000  $\mu$ moles of soy PE in a methylene chloride slurry under a nitrogen atmosphere at 70°C overnight. The unreacted succinic anhydride was filtered  
35 off. The methylene chloride was evaporated and the succinic

- 13 -

acid modified PE was purified by column chromatography. 500  $\mu$ moles of the succinic acid modified PE was dissolved in benzene, and 500  $\mu$ moles of 1,1 carbonyl diimidazol were added. The solution was heated at 60°C, until the bubbling had ceased. 750  $\mu$ moles of the polyethylene glycol monomethylether (molecular weight 252) were added to the solution, and the mixture was heated for 3 hours under a nitrogen atmosphere at 70°C. The purified product was then recovered as described in Example 1.

10 Example 8. Soy phosphatidylethanolamine succinyl tetraethylene glycol (molecular weight 194)

The soy PE succinic acid derivative was prepared as described in Example 7. 650  $\mu$ moles of the compound were dissolved in benzene and 650  $\mu$ moles of 1,1 carbonyl diimidazole were added. The solution was heated at 60°C, until the bubbling had ceased. To the solution were added 650  $\mu$ moles of tetraethylene glycol. The reaction was heated at 70°C under a nitrogen atmosphere for 3 hours. The reaction was recovered as described in Example 1. The yield of the column-purified material was 37%.

20 Example 9. Soy phosphatidylethanolamine glutaryl polyethylene monomethylether (molecular weight 252)

One of the disadvantages of using the succinic anhydride as a linking agent is the high melting point of the anhydride that prevents a homogeneous reaction mixture. In using glutaric anhydride, many of these disadvantages can be addressed. In particular, 1 mole of the polyethylene glycol was heated with 2 to 3 moles of the glutaric anhydride in a N<sub>2</sub> atmosphere at 75°C in the absence of any solvent. At the end of the reaction as determined by thin-layer chromatography, the reaction mixture was dissolved in methylene chloride/methanol 1:1 (v/v) and recovered as described for the succinyl material as described in Example 1. In this particular example, 7.5 g of polyethylene glycol monomethylether (molecular weight 252) and 5 g of glutaric

- 14 -

- anhydride were placed in a reaction vessel and heated to 75°C, with magnetic stirring, for 3 hours under a N<sub>2</sub> atmosphere. The glutaryl polyethylene glycol compound was purified by column chromatography. 1200  $\mu$ moles of 1,1
- 5 carbonyl diimidazole were added to 1500  $\mu$ moles of the glutaryl polyethylene glycol compound dissolved in benzene. The solution was heated at 60°C, until the bubbling had ceased. This solution was added to 1127  $\mu$ moles of dry soy PE, and the reaction mixture was heated for 3 hours at 70°C.
- 10 The mixture was extracted and purified as described in Example 1. The yield of pure product was 67%.

Examples 10-14 illustrate that the phospholipids of the invention are useful in solubilizing water-insoluble drugs, oils and fragrances, to provide aqueous solutions.

15 Example 10.

- The anticancer drug, Taxol (an experimental drug of the National Cancer Institute which inhibits the ability of cells to divide) is insoluble in water. 8  $\mu$ moles of Taxol (6.5 mg) were dissolved in methylene chloride with 72  $\mu$ moles of soy
- 20 phosphatidylethanolamine succinyl polyethylene glycol monomethylether (average molecular weight 550). The solution was taken to dryness and pumped on by a high vacuum. The dried material was hydrated with 1 ml of 10 mM Tris (pH 8.5) and vortexed at room temperature. The resulting solution was
- 25 optically clear. The solution was then adjusted with concentrated dextrose, to bring the dextrose concentration to 0.3M; thus making the sample suitable for intravenous injection.

Example 11.

- 30 Pentobarbital is a barbiturate that is insoluble in water at physiological pH as the acid form. It is the water-insoluble form that exerts its therapeutic activity.



- 15 -

A standard solution sodium pentobarbital is stable nly at high pH and was adjusted with dilute HCL to pH 3. The precipitated pentobarbital was removed by filtration. 40 umoles of the pentobarbital were dissolved in methylene chloride with 60 umoles of the soy phosphatidylethanolamine succinyl polyethylene glycol monomethylether (molecular weight 252). The solution was taken to dryness and then pumped on by high vacuum. The dried material was hydrated with 2 ml of 10 mM Tris (pH 8.5), to form an optically clear solution. The solution was adjusted with concentrated dextrose to give a 0.3M solution suitable for intravenous injection.

Example 12.

The anticancer drug, hexamethylmelamine, is water-insoluble. 2 mg of hexamethylmelamine and 70 umoles of soy phosphatidylethanolamine succinyl polyethylene glycol monomethylether (average molecular weight 550) were dissolved in methylene chloride. The solution was taken to dryness and then pumped on by high vacuum. The solution was hydrated with 1 ml of 10 mM Tris (pH 8.5), to give an optically clear solution. The solution was adjusted with concentrated dextrose to give a final dextrose concentration of 0.3M suitable for intravenous injection.

Example 13.

Another property of the phospholipids of the invention is their ability to act as exceedingly powerful surfactants for cosmetic ingredients, such as oils and petroleum jelly. As an illustration of such ability, 1.6 g of petroleum jelly and 160 mg of soy phosphatidylethanolamine succinyl polyethylene glycol monomethylether (molecular weight 252) were dissolved in hexane and taken to dryness and pumped on at high vacuum. To the dried material were added 4 ml of 10 mM Tris (pH 8.5). The hydrated solution was then sonicated with a Branson W-375 sonifier at 40°C for 2 minutes. The resulting solution was opaque, but had excellent flow characteristics. When applied

- 16 -

to the skin, there was an immediate and noticeable cooling sensation, due to the evaporation of water, and a pleasing tactile sensation.

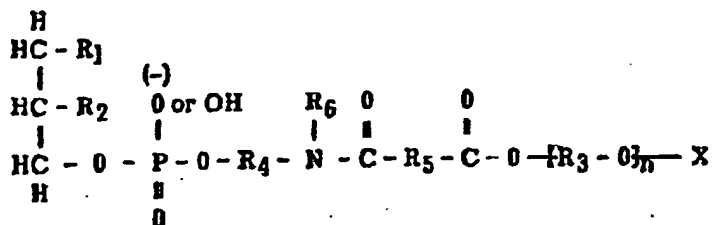
Example 14.

- 5        Another example of the use of the compounds of the invention in the cosmetic field is the solubilization of water-insoluble fragrance oils in an aqueous environment. For example, 0.250 ml of a fragrance oil and 200  $\mu$ moles of soy phosphatidylethanolamine glutaryl polyethylene glycol
- 10 monomethylether (molecular weight 252) were dissolved in 0.5 ml of ethanol. The solution was then diluted with 40 ml of water. The resulting solution was optically clear and retained the fragrance aroma.

- 17 -

## CLAIMS

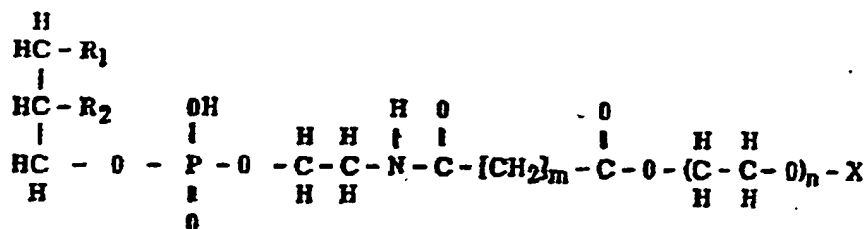
1. A synthetic phospholipid compound having the structural formula:



wherein  $R_1$  and  $R_2$  each represent hydrogen or an organic acyl radical;  $R_3$  represents a  $C_2$  or  $C_3$  alkylene radical;  $X$  represents hydrogen or an alkyl radical;  $R_4$  represents a  $C_2$  to  $C_{10}$  alkylene radical;  $R_5$  represents an organic linking radical;  $R_6$  represents hydrogen or a  $C_1$  to  $C_4$  alkyl radical; and  $n$  represents a number of from 0 to 200.

2. A phospholipid compound according to claim 1 wherein  $R_1$  and  $R_2$  each represent a  $C_2$  to  $C_{20}$  fatty-acid radical.
3. A phospholipid compound according to claim 1 wherein  $R_1$  and  $R_2$  are organic radicals derived from soybeans.
4. A phospholipid compound according to claim 1, 2 or 3 wherein  $R_5$  represents a polymethylene radical.
5. A phospholipid compound according to claim 4 wherein  $R_5$  represents a  $C_1$  to  $C_4$  methylene radical.
6. A phospholipid compound according to any one of the preceding claims wherein  $n$  represents a number from 2 to 20.
7. A synthetic phospholipid compound according to claim 1 which is a soy phosphatidylethanolamine succinyl or glutaryl polyethylene glycol compound.

8. A synthetic phospholipid compound having the structural formula:



wherein  $R_1$  and  $R_2$  each represent a  $C_2$  to  $C_{20}$  hydrocarbon organic acyl radical; X represents hydrogen or a methyl radical; m represents a number of from 1 to 4; and n represents a number of from 0 to 200.

9. A method of preparing a phosphatidylalkanolamine polycarboxyl polyalkylene glycol compound, which method comprises reacting a phosphatidylalkanolamine, a polycarboxylic acid or derivative thereof and a polyalkylene glycol.

10. A method according to claim 9 which comprises reacting a dicarboxylic acid anhydride with a poly C<sub>2</sub> or C<sub>3</sub> alkylene glycol, to form the carboxyl poly C<sub>2</sub> or C<sub>3</sub> alkylene glycol compound and thereafter reacting the carboxyl poly C<sub>2</sub> or C<sub>3</sub> alkylene glycol compound with a phosphatidylethanolamine.

11. A method according to claim 9 or 10 wherein the polyalkylene glycol is polyethylene glycol having a molecular weight of from 150 to 3000.

12. A method according to claim 9, 10 or 11 wherein the polycarboxylic acid derivative is a C<sub>2</sub> to C<sub>7</sub> dicarboxylic acid anhydride.

- 19 -

13. A method according to any one of claims 9 to 12 wherein the phosphatidylalkanolamine is phosphatidylethanolamine.
14. A method according to claim 13 wherein the phosphatidylethanolamine is derived from crude soy lecithin.
15. A method according to any one of claims 9 to 14 which includes recovering the phosphatidylalkanolamine polycarboxyl polyalkylene glycol by extracting with acetone.
16. A method according to claim 9 which comprises reacting a dicarboxylic acid anhydride with a phosphatidylethanolamine, to form the phosphatidylethanolamine carboxylic acid compound and thereafter reacting the phosphatidylethanolamine carboxylic acid compound with a poly C<sub>2</sub> or C<sub>3</sub> alkylene glycol.
17. A method according to any one of claims 9 to 16 wherein the dicarboxylic acid derivative is succinyl acid anhydride or glutaric acid anhydride.
18. A method of solubilizing, in an aqueous environment, a water-insoluble compound, which method comprises mixing a solubilizing amount of a hydrated phospholipid compound as claimed in any one of claims 1 to 8 with water and a water-insoluble compound.
19. An aqueous composition which comprises water, a micelle-forming amount of a synthetic phospholipid as claimed in any one of claims 1 to 8 and a water-insoluble compound.
20. An aqueous composition according to claim 19, which solution has micelles therein of less than 20 nm (200 Angstroms) in size.
21. An aqueous composition according to claim 19 or 20 which comprises a therapeutic drug, petroleum jelly or a fragrance oil as the water-insoluble compound.

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5325-105.30F	9,966 05-13-92 12:03p	✓ 5325-105.30L <i>Approved</i>	33,789 07-13-92 03:35p
5325-105.30Q	35,373 07-19-92 04:31p	✓ 5325-105.31 <i>Approved</i>	26,558 10-01-91 04:33p
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Sears

lipids are analogues of gangliosides, and on Allen et al. as teaching the ability of gangliosides, in a mole ratio of 5-20 percent, to extend the circulation time of liposomes, and further for the teaching that liposomes with gangliosides may be used for tumor targeting.

#### A. The Prior Art

The Sears patent describes a novel phospholipid analog derivatized with polyethylene glycol through alkylene radical having 2-10 carbon atoms. The Sears patent deals primarily with micellar, as opposed to liposomal, structures which can be formed from lipids with hydrophilic head groups, e.g., PEG-derivatized phospholipids. The only reference to liposomes in the Sears patent is with regard to using the derivatized lipids to stabilize natural phospholipid structures, such as small unilamellar vesicles (SUVs), which are naturally unstable (column 4, 10-18). SUV's are typically defined as having vesicle average sizes between about 0.02 and 0.07 microns.

Sears notes that by covalently attaching a hydrophilic polymer, such as polyethylene oxide polymers, to phosphatidylethanolamine, a phospholipid analog to ganglioside is essentially formed.

Allen et al. and earlier U.S. Patent No. 4,837,028 to Allen disclose a liposome formulation composed of predominantly rigid lipid components and 5-15 mole percent of the ganglioside GM<sub>1</sub>, and characterized by an extended liposome circulation time in the blood. The general conclusions which can be drawn from the two Allen patents are:

(a) Enhanced circulation time in liposomes was specific to two glycolipids: GM<sub>1</sub> and hydrogenated phosphatidylinositol (HPI). A large number of other glycolipids and/or

charged lipids which were tested failed to give comparable blood circulation times (Tables 3-6 in the '016 patent);

(b) For both GM<sub>1</sub> and HPI, enhanced circulation time was dependent on the presence of relatively rigid lipid components, i.e., lipid components with high phase transition temperatures; and

(c) The best blood/RES ratios observed for optimal GM<sub>1</sub> (or HPI) liposome compositions, as measured 24 hours post injection, were between about 0.4-0.9 (Table 6 of the '016 Patent).

#### B. Differences between the Claimed Invention and the Prior Art

The differences between the Sears and Allen teachings have been discussed in the Applicants' Amendment filed 10/30/91 and were reviewed in the 3/10/92 Interview. The following points were stressed:

(1) Sears notes that by covalently attaching a hydrophilic polymer, such as polyethylene oxide polymers, to phosphatidylethanolamine, a phospholipid analog to ganglioside is essentially formed. However, this passage refers only to the well known ability of virtually any water soluble moiety, including sugar moieties, and hydrophilic polymer moieties, to confer surfactant properties on a derivatized phospholipid. The surfactant property is evidenced by the ability of the derivatized lipids to form micelles (rather than liposomes) when dispersed in an aqueous solvent.

(2) The ability of GM<sub>1</sub> to extend liposome circulation time is highly specific for GM<sub>1</sub> among a large number of other gangliosides and glycolipids which were examined for their ability to extend blood circulation time. This is seen, for example, from Table 1 in the Allen patent, where substituting asialoganglioside (ASGM<sub>1</sub>) for GM<sub>1</sub> in liposomes (composition 19



vs. composition 17) largely abolishes the enhanced circulation time produced by GM<sub>1</sub> enhancement. Similarly, several other glycolipids, including monogalactosyl-stearate sulfatide (SULF), ceramide trihexose (CTRI), digalactosyl diglyceride (DGDG), monogalactosyl diglyceride (MGDG), galactosyl cerebroside (GAL), globosides (GLOB), and glucocerebroside (GLU) (Table 3) and modified gangliosides GM<sub>2</sub>, GM<sub>3</sub>, GD<sub>1a</sub>, GT<sub>1b</sub> (Table 4) were largely ineffective or substantially less effective than GM<sub>1</sub> in extending liposome blood circulation time.

3. A variety of evidence collected in support of the present invention indicates that the mechanism by which GM<sub>1</sub> extends blood circulation time is different from that by which PEG extends circulation time.

In the 3/10/92 Interview, the Examiner indicated that the combination of Sears and Allen et al. could be overcome by data showing that the properties and mechanisms by which PEG and GM<sub>1</sub> liposomes extend blood circulation time are different. Part I of the Martin Declaration, filed 05/13/92, discussed the following differences between PEG-containing liposomes and GM<sub>1</sub>-containing liposomes:

1. The effect of GM<sub>1</sub> on liposome circulation time is related to the type and number of sugar residues in glycolipid components of the liposomes (as discussed in point 2 above).

By contrast, the long circulation time for liposomes achieved in the present invention do not involve glycolipids, and is thus independent of any effect related to type and number of sugar residues carried on the liposome surfaces.

2. The enhancement of blood circulation by GM<sub>1</sub> is dependent on the presence in liposomes of predominantly membrane-rigidifying vesicle-forming components, such as sphingomyelin (SM) or neutral phospholipids with saturated

acyl chains. For example, comparing compositions 3 and 8 in Table 1 in Allen, it is seen that substituting DSPC (a saturated acyl chain PC) for unsaturated egg PC (PC) in a GM<sub>1</sub>-containing liposome formulation increases blood/RES ratios nearly 12-fold.

By contrast, the data presented in Table 5 of the present application indicate that the degree of saturation of membrane phospholipid components has no systematic effect on blood/RES ratios.

3. Experiments carried out jointly by LTI and Dr. Allen have shown that addition of the negatively charged phospholipid phosphatidylglycerol (PG) to GM<sub>1</sub> liposomes significantly reduces measured blood circulation time, with the extent of reduction being dependent on the molar concentration of PG in the liposomes.

By contrast, the percent PG in the PEG-liposomes of the present invention had no appreciable effect on blood circulation times (Example 8, discussed with reference to Figure 7).

4. The best blood/RES ratios observed for optimal GM<sub>1</sub> liposome compositions, as measured 24 hours post injection, were between about 0.4-0.9 (Table 6 of the Allen et al. patent). This is in contrast to the present invention, in which blood/RES ratios at 24 hours as high as 4 were observed (Table 3 in the present application).

It is noted that the blood/RES ratios reported in the '016 patent were measured by methods identical to those used in the present application. As noted in the Martin Declaration, the Applicants have worked closely with Dr. Allen at the University of Alberta for several years and have standardized the methods for measuring blood/RES ratios used by both the LTI and the University of Alberta groups.

#### C. Analysis

In summary, Sears suggests only that gangliosides (or any other surfactant) and PEG-phospholipids would be expected to have similar micelle-forming surfactant properties in vitro. In view of specificity of the GM<sub>1</sub> effect observed by Allen, and the different mechanisms by which GM<sub>1</sub> and PEG appear to extend blood circulation time in vivo, it cannot be considered obvious to substitute the PEG-phospholipid taught by Sears for GM<sub>1</sub> in the long-circulating liposome formulation taught by Allen.

The remaining references to Perez-Solar and Forssen are representative of a number of references showing anti-neoplastic agents encapsulated in liposomes, for use in treating tumors. Neither of these references show or suggest liposomes with long-circulation times, nor how such liposomes could be produced.

In addition, none of the cited prior art references show or suggest the possibility of achieving the improvement in tumor-treatment results achieved in the present invention. As noted above, the results include:

(a) virtually complete inhibition of tumor growth in animals for a variety of solid tumors, and using PEG-liposomes containing different anti-tumor compounds;

(b) a dramatic increase in survival time in treated animals having a variety of solid tumors; and

(c) a dramatic improvement, in terms of patient comfort and stabilization of hemoglobin levels, in AIDS patients treated for Kaposi's Sarcoma.

For these reasons, the Applicants respectfully submit that Sears in view of Allen and Perez-Solar and Foressen do not render obvious Applicant's invention as claimed and urge the Board to overturn the Examiner's rejection.

Respectfully submitted,